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PD-1$^+$ memory phenotype CD4$^+$ T cells expressing C/EBPα underlie T cell immunodepression in senescence and leukemia

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Summary

Although altered T cell function takes part in immunosenescence, the mechanisms remain uncertain. Here we identify a bona fide age-dependent PD-1$^+$ memory phenotype (MP) CD4$^+$ T cell subpopulation, which hardly proliferates in response to TCR-stimulation and produces abundant osteopontin at the cost of typical T-cell lymphokines. These T cells show an impaired repopulation in Rag2-/- mice, whereas a homeostatic proliferation in γ-ray irradiated mice is retained. These T cells also reveal a unique molecular signature, including a strong expression of C/EBPα normally expressed in myeloid-lineage cells with diminished c-Myc and Cyclin D1. Transduction of *Cebpa* in regular CD4$^+$ T cells inhibited the TCR-mediated proliferation with *c-Myc* and *Cyclin D1* repression and caused a striking activation of *Spp1*encoding osteopontin with concomitant repression of T-cell lymphokine genes. Although these T cells are gradually increased with age and become predominant at senescent stage in normal mice, the generation is robustly accelerated during leukemia. In both conditions, their predominance is associated with the diminution of specific CD4$^+$ T cell response. The results suggest that global T-cell immunodepression in senescence and leukemia is attributable to the increase in PD-1$^+$ MP CD4$^+$ T cells expressing C/EBPα.

¥body

Introduction

The elderly may show a substantial diminution in specific immune response against infection, a reduced efficacy for vaccination and a proinflammatory trait, being called immunosenescence (1, 2). In T-lineage cells, a most prominent effect of aging is thymic involution, resulting in the production and export of fewer T cells (3). Total numbers of peripheral T cells, however, are unaffected by aging in both humans and mice, owing in part to the homeostatic proliferation of memory phenotype (MP) T cells (4-6); consequently, T cell population shows a progressive shift from naïve to MP cells with age. Such a shift in T cell composition is considered to contribute significantly to immunosenescence. This may result in contraction of the T cell repertoire, leading to an increased incidence of poor responsiveness to new antigens (7). It is also recognized that $CD4^+$ T cells in the elderly are qualitatively altered, including a number of defects in the TCR-mediated signaling pathways, reduced immunological synapse formation with antigen-presenting cells as well as cognate helper function for B cells, and altered lymphokine production patterns (8-11). These effects may be attributable primarily to the cellular changes of T cells rather than to the host environment (12).

The homeostatic maintenance of MP T cells for prolonged periods may involve multiple factors, including environmental antigens such as commensal bacteria, low-affinity self-ligands and homeostatic cytokines (4, 13, 14). The eventual fates of MP T cells, however, remain elusive. In most somatic tissue cells, programmed cell differentiation is tightly coupled with the control of cell proliferation, leading to the

terminal differentiation with loss of proliferation capacity and limited life span. In myeloid-lineage cells, for instance, a bZIP family transcription factor C/EBPα plays a crucial role in controlling the homeostatic differentiation and the quiescence of terminally differentiated cells (15, 16). Expression of *Cebpa*, however, is repressed during T-lineage cell commitment from hematopoietic progenitors, and maturated T cells hardly express C/EBPα (17). Currently, it remains uncertain how the balance between proliferation and quiescence of MP T cell clones is controlled to maintain the homeostasis for prolonged periods.

In current study, we identify a bona fide age-dependent MP $CD4^+$ T cell population defined by a constitutive expression of PD-1, which is only transiently induced on activation in regular T cells (18). The PD-1^+ MP $CD4^+$ T cells hardly proliferate in response to TCR stimulation but produce large amounts of a proinflammatory cytokine, osteopontin (OPN), at the cost of typical T-cell lymphokines. We suggest that the functional features of these cells are attributable in part to an unusual expression of C/EBPα. Besides in senescence, the generation of equivalent PD-1^+ MP $CD4^+$ T cells is robustly accelerated during leukemia. We provide evidence that the predominance of these unique T cells underlies global depression of T cell immune response both in senescence and during leukemia.

Results

Identification of Age-dependent PD-1$^+$ MP CD4$^+$ T Cells with Defective TCR-mediated Proliferation

We found that increasing proportions of splenic CD4$^+$ T cells in normal B6 mice constitutively expressed PD-1 as they aged (Fig. 1A, upper). The PD-1$^+$ CD4$^+$ T cells were confined to a CD44^{high} CD62L^{low} (MP) population, and the majority of them exhibited CD69 with little CD25 expression (Fig. 1A, lower). Purified CD4$^+$ T cells from aged mice showed a significantly diminished TCR-mediated proliferation compared with those from young mice (Fig. 1B). Among the CD4$^+$ T cells in aged mice, however, both naïve and PD-1$^-$ MP populations revealed the response comparable with those from young mice; in stark contrast, PD-1$^+$ MP population showed no detectable proliferation, despite normal expression of αβTCR/CD3 (Fig. 1B). They showed no anexin V staining and thus were not dying cells in vivo (Fig. S1A). Costimulation with anti-CD28 antibody or IL-2 only marginally restored the proliferation (Fig. S1B). Furthermore, these T cells revealed negligible production of typical T-cell lymphokines in response to optimal TCR stimulation (Fig. S1C). PD-1$^+$ MP CD4$^+$ T cells showed a severely impaired repopulation capacity in Rag-2$^{-/-}$ mice (Fig. 1C, upper left). Because robust T cell expansion in Rag-2$^{-/-}$ recipients mostly represents the response to exogenous antigens such as commensal bacteria (13), these T cells are suggested to be defective in TCR-mediated proliferation in vivo as well. On the other hand, PD-1$^+$ MP CD4$^+$ T cells revealed a homeostatic expansion comparable with the PD-1$^-$ T cells in γ-ray irradiated recipients (Fig. 1C, lower left); in agreement, a significant proportion of

PD-1$^+$ CD4$^+$ T cells expressed IL-15R and/or IL-7R (Fig. 1C, right). Although PD-1$^+$ cells represent a rare population in CD4$^+$ T cells until 6 months of age, they are increased linearly throughout later stages, eventually becoming a predominant population at senescent stage (Fig. 1D). These T cells are found in most lymphoid tissues of aged mice, except for the peripheral blood (Fig. S1D), and the profile of TCR-Vβ chain usage is unchanged (Fig. S1E). We found that PD-1$^+$ MP CD4$^+$ T cells overexpressed *Cd121b* by DNA microarray analysis (see below) and confirmed that a portion of these T cells selectively expressed CD121b (Fig. S2A). PD-1$^{-/-}$ CD121b$^+$ CD4$^+$ T cells remained defective in TCR-mediated proliferation, suggesting that PD-1 expression might be irrelevant for the effect (Fig. S2A). Stimulation of PD-1$^+$ MP CD4$^+$ T cells with PMA plus ionomycin bypassing TCR-stimulation failed to induce significant proliferation either, despite nearly normal ERK activation (Fig. S2B). Finally, PD-1$^+$ MP CD4$^+$ T cells completely depleted of CD25$^+$ cells showed no TCR-mediated proliferation or inhibitory effect on the proliferation of normal CD4$^+$ T cells (Fig. S2C). These results suggest that the defect in TCR-mediated proliferation may be intrinsic.

Unique Genetic Signature and Potent OPN Production

Given the age-dependency, we examined the possibility that PD-1$^+$ MP CD4$^+$ T cells might represent "senescent" T cells, with the use of senescence-associated β-galactosidase (SA-β-Gal) (19). Although CD4$^+$ T cells from 2 month-old mice rarely showed SA-β-Gal staining, a significant proportion of MP, but not naïve, CD4$^+$ T cells from 18 month-old mice revealed positive staining; however, there was no significant

difference in the frequencies and the intensities between PD-1$^-$ and PD-1$^+$ MP fractions (Fig. 2A). We then compared the global gene expression profiles by DNA microarray analysis (Table S1). We selected 37 genes from the data and compared their expression among naïve, PD-1$^-$ MP and PD-1$^+$ MP fractions of CD4$^+$ T cells by qRT-PCR (Fig. 2B). 27 genes showed a markedly increased expression in a PD-1$^+$ MP population, of which 17 genes including *Pdcd1* and *Cd121b* were overexpressed rather selectively, whereas the expression of 10 genes was decreased. PD-1$^+$ MP CD4$^+$ T cells were found only minimally in young mice (see Fig. 1A); however, the genetic signature of these cells highly coincided with those in aged mice (Fig. 2C), indicating that these T cells began to emerge early in life. At the top of the overexpressed genes was *Spp1* encoding OPN (also called Eta-1). In agreement, PD-1$^+$, but not PD-1$^-$, MP CD4$^+$ T cells spontaneously secreted significant amounts of OPN. Furthermore, these cells showed a prominently enhanced OPN secretion upon TCR stimulation (Fig. 2D, upper); this was associated with a robust increase in *Spp1* transcripts, whereas the induction of *Il-2*, *Ifn-γ* and *Il-4* was significantly compromised (Fig. 2D, lower). These T cells additionally showed abundant transcripts of *Sostdc-1*, encoding a secreted antagonist of anti-inflammatory bone morphogenic factor (Fig. 2D).

Unusual Expression of C/EBPα: A Role in the Unique Functional Features

PD-1$^+$ MP CD4$^+$ T cells also revealed a remarkably altered expression of several transcription-related genes (see Fig. 2B). Immunoblotting analysis confirmed that these cells strongly expressed C/EBPα, VDR, OcaB and Bcl6, whereas Satb1, c-Myc and

Cyclin D1 were hardly detectable (Fig. 3A). Because abundant expression of C/EBPα was particularly unexpected, we examined the effects of forced *Cebpa* expression in regular $CD4^+$ T cells. Young $CD4^+$ T cells were stimulated with anti-CD3 plus anti-CD28 antibodies and infected with *Cebpa*-containing MIG. Sorted GFP^+ cells exhibited a largely comparable level of *Cebpa* transcripts with natural PD-1^+ MP $CD4^+$ T cells. The *Cebpa*-expressing $CD4^+$ T cells revealed a significant repression of *c-Myc*, *Cyclin D1*, *Il-2* and *Il-4*, with undetectable proliferation (Fig. 3B). On the contrary, these T cells revealed a robust activation of *Spp1*, while *Satb1*transcripts were unaffected (Fig. 3B). Although the detection of these proteins was unfeasible due to very limited cell numbers, the results suggest that the expression of C/EBPα may be responsible at least in part for the unique functional features of PD-1^+ MP $CD4^+$ T cells.

Rapid and Robust Increase in PD-1^+ MP $CD4^+$ T Cells during Leukemia

A global diminution of specific T cell response often develops in malignancy. To investigate the possible involvement of PD-1^+ MP $CD4^+$ T cells, we transplanted *bcr/abl*-induced leukemia cells (BA-1) into young B6 mice. Within 2 weeks as frank leukemia developed, the recipients revealed a marked increase in PD-1^+ MP cells, preferentially in the $CD4^+$ T cell population (Fig. 4A). The $CD4^+$T cells from leukemic mice showed a significantly diminished TCR-mediated proliferation; this was attributable to the defective response specifically of PD-1^+ MP cells (Fig. 4B). Also, PD-1^+ MP $CD4^+$ T cells secreted by far greater amounts of OPN than PD-1^- $CD4^+$ T cells on TCR stimulation, whereas the production of T-cell lymphokines was

compromised (Fig. 4B). Similar results were obtained with an independent leukemia cell line. Because PD-L1 was hardly or only marginally expressed on these leukemia cells (Fig. S3A), it did not appear to be relevant for the effects. Spa-1$^{-/-}$ mice, which spontaneously developed various leukemia (20, 21) also showed a remarkable increase in PD-1^{+} MP CD4^{+} T cells after leukemia development (Fig. S3B, C). DNA microarray cluster and qRT-PCR analysis revealed highly coincided gene expression profiles between the PD-1^{+} MP CD4^{+} T cells in leukemic and normal aged mice (Fig. 4C). Kinetic analysis indicated that the proportions of PD-1^{+} MP CD4^{+} T cells were rapidly increased with the systemic spread of leukemia, and this was inversely correlated with the diminution of TCR-mediated CD4^{+} T cell proliferation (Fig. 4D).

Emergence of PD-1^{+} CD4^{+} T MP cells is correlated with the clonal T-cell immunodepression in senescence and during leukemia.

Tg mice of OVA-specific TCR (OT-II) at Rag2$^{-/-}$ background only barely developed MP T cells and accordingly few PD-1^{+} cells even at the age of 12 months (Fig. 5A, upper). The Rag2$^{-/-}$ OT-II cells revealed no diminution of, but rather an increase in, the OVA-specific proliferation compared with those from young mice (Fig. 5A, lower). Similarly, OT-II/Rag2$^{-/-}$ mice transplanted with BA-1 cells developed few PD-1^{+} MP CD4^{+} T cells with no diminution of OVA-specific T cell response, despite massive leukemia burden (Fig. 5A). In contrast, aged OT-II Tg mice at B6 background revealed a marked increase in PD-1^{+} MP CD4^{+} T cells including Vα2^{+} OT-II T cells (Fig. 5B, upper). Although PD-1^{-} OT-II T cells showed significant OVA-specific proliferation,

PD-1$^+$ MP OT-II T cells revealed no detectable proliferation (Fig. 5B, lower). Transplantation of BA-1 cells in young OT-II/B6 mice caused even more prominent increase in PD-1$^+$ MP OT-II T cells with a profound diminution of OVA-specific response (Fig. 5B). These results suggest at the clonal level that the generation of PD-1$^+$ cells are concomitant with the development and expansion of MP CD4$^+$ T cells, and that their increase underlies T cell immunodepression with age and during leukemia.

Discussion

Naturally arising MP $CD4^+$ T cells appear to be composed of heterogeneous populations, only a fraction of them being functional memory T cells (4). In current study, we have identified a distinct population within MP $CD4^+$ T cells, which constitutively expresses PD-1 and partly CD121b and hardly proliferates nor produces typical T-cell lymphokines on TCR stimulation in vitro. PD-1^+ MP $CD4^+$ T cells show a severely impaired repopulation capacity in Rag2-/- mice, in which regular T cells exhibit a robust expansion in response to exogenous antigens, in particular commensal bacteria (13). The results suggest that these T cells are defective in antigen-specific response in vivo, although an additional possibility for the selective susceptibility to host NK cells as reported in certain $CD4^+$ T cells (22) remains to be examined. In contrast, PD-1^+ MP $CD4^+$ T cells reveal a repopulation in irradiated B6 recipients comparably with PD-1^- $CD4^+$ T cells, suggesting that these cells respond to homeostatic cytokines. Thus, PD-1^+ $CD4^+$ T cells may represent a unique MP cell population, which are capable of homeostatic proliferation with little contribution to antigen-specific immune response based on the clonal expansion.

Importantly, PD-1^+ MP $CD4^+$ T cells are steadily increased and accumulated with age in normal mice, becoming a predominant population at the senescent stage. Despite the age-dependent increase, analysis of SA-β-Gal expression argues against a possibility that these cells simply represent a classic cell-senescence; rather, the generation of these cells is suggested to be concomitant with the homeostatic proliferation of PD-1^- MP $CD4^+$ T cells. The generation of PD-1^+ MP $CD4^+$ T cells

appears to be associated with a unique genetic reprogramming. A most notable molecular feature is a strong expression of C/EBPα, which is normally expressed in nonlymphoid cells including myeloid cells (15). C/EBPα shows a potent antiproliferative effect either reversibly by interacting with Cdk inhibitors or irreversibly by repressing *c-Myc* (15, 23). In agreement, a forced expression of *Cebpa* in regular $CD4^+$ T cells resulted in the loss of TCR-mediated proliferation capacity with the repression of *c-Myc* and *Cyclin D1*. Furthermore, it induced a prominent activation of *Spp1* in concomitant with the inhibition of T-cell lymphokine gene activation, although the role of endogenous C/EBPα in natural PD-1^+ MP $CD4^+$ T cells remained to be proved. Interestingly, an artificial expression of *Cebpa* in thymic pre-T cells is reported to redirect their differentiation into myeloid cells with the repression of T cell-specific genes (17). In this aspect, it is noted that PD-1^+ MP $CD4^+$ T cells show a profoound repression of *Satb1*, a T cell-specific gene crucial for the development and function of T cells (24, 25). Satb$1^{-/-}$ T cells are also reported to show a enhanced *Pdcd1* expression (25); thus, the constitutive PD-1 expression may be due in part to *Satb1* repression. Unlike “exhausted” $CD8^+$ T cells generated during chronic viral infection (26), current results suggest no relevant involvement of PD-1 in the defective proliferation of PD-1^+ MP $CD4^+$ T cells; however, other possible effects of PD-1 ligated by PD-L remain to be seen.

PD-1^+ MP $CD4^+$ T cells with equivalent genetic and functional features to those in senescence are robustly increased during leukemia, suggesting that the senescence-related cellular and genetic changes in $CD4^+$ T cells can be rapidly

accelerated in malignancy. In humans, leukemia patients often show a profound T cell immunodepression; a recent report indicates that the effects may be due in part to the changes in the gene expression of T cells upon interaction with leukemia cells (27). Intriguingly, OT-II Rag2-/- mice hardly developed this population, and the OA-specific responsiveness of these mice was well sustained with age and during leukemia. In contrast, OT-II B6 mice revealed a marked increase in the PD-1$^+$ MP cell population similar to normal B6 mice in both conditions, resulting in a profound diminution of OVA-responsiveness. The reasons for the difference remain to be investigated; it may be possible that endogenous TCRs possibly expressed on B6 OT-II T cells are required for the development of PD-1$^+$ MP cells. Nonetheless, the results indicate that the increase in PD-1$^+$ MP cell proportion is directly associated with the progression of CD4$^+$ T cell immunodepression in senescence and during leukemia.

OPN is a potent proinflammatory cytokine produced by many cell types, including macrophages, dendritic cells and activated Th1 cells, and plays a significant role in inflammatory diseases (28, 29). Current results suggest that PD-1$^+$ MP CD4$^+$ T cells may also serve as a potent OPN producer, either spontaneously or inductively via TCR stimulation. The OPN production by these T cells seems to be controlled by C/EBPα, whereas that by Th1 cells is under the regulation by T-bet (30). Recently, T cell-intrinsic OPN is reported to function as an autocrine prosurvival factor for CD4$^+$ T cells in vivo (31); thus, OPN may also contribute to the maintenance of PD-1$^+$ MP CD4$^+$ T cells in vivo. In addition, OPN may affect the growth and invasion of malignant cells, directly or indirectly by recruiting host inflammatory cells (32, 33). As such, these T

cells may contribute to a proinflammatory trait in the elderly as well as tumor progression. Manipulation of PD-1^{+}MP CD4^{+} T cells may provide a clue for restoring the adverse immune traits in senescence and malignancy.

Materials and Methods

Refer to SI Text for detailed materials and methods.

Mice. The C57BL/6 (B6), B6 (CD45.1), Rag2$^{-/-}$, SPA-1$^{-/-}$, PD-1$^{-/-}$ and OT-II Tg mice, all at B6 background, were maintained in specific pathogen-free conditions at the Center of Laboratory Animals of Kyoto University under the University guidelines.

Flow cytometry and cell culture. Multicolor flow cytometric analysis and cell sorting were performed with FACSCalibur and FACSAria (BD Biosciences). Cell proliferation was assessed by the incorporation of ^{3}H-thymidine, and cytokines were measured with ELISA.

Cell transfers. Purified CD4^{+} T cell subpopulations were injected intravenously into Rag2$^{-/-}$ or γ-ray (6 Gy)-irradiated B6 (CD45.1) mice (3×10^{6} cells/head). BA-1 leukemia cells were transplanted intravenously into normal B6 mice ($1\sim5 \times 10^{6}$ cells/head).

Quantitative RT-PCR analysis. Real-time PCR was performed with LightCycler SYBR Green I marker kit on a LightCycler instrument (Roche).

SA-β-Gal staining. T cells were plated on poly-L-lysine-coated cover slips, fixed with glutaraldehyde (0.5% in PBS), washed with Mg2^{+}-containing PBS, and stained with X-Gal in PBS containing $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and Mg2^{+}.

Gene transduction. A *Cebpa* cDNA, provided by Dr. Iwama (Tsukuba University, Tsukuba, Japan), was subcloned into the retroviral plasmid (pMCs IRES GFP, pMIG) provided by Dr. Kitamura (University of Tokyo, Tokyo, Japan). Recombinant retrovirus was produced in Plat-E packaging cells.

DNA microarray and clustering analysis

Comprehensive DNA microarray analysis was performed with 3D-Gene™ (Toray Industries, Inc., Tokyo, Japan). Microarrays were scanned with the ScanArray Lite Scanner (Perkin-Elmer) and analyzed with the Cluster 3.0.

Statistical analysis

Statistical analysis was performed with the two-tailed Student t test.

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Author contribution:

K.S. principally contributed to general experiments; Y.N. performed the experiments on leukemia model; H.M. contributed to the plasmid preparations and discussions; Y.H. contributed to the SA-β-Gal staining and discussions; and N.M. provided overall design of the work and wrote a manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Figure Legends

Fig. 1. Age-dependent increase in PD-1$^+$ MP CD4$^+$ T cells with defective TCR-mediated proliferation. (A) Spleen cells from normal B6 mice at the various ages were three-color analyzed with the indicated antibodies. (B) CD4$^+$ T cells from 2-month-old (open column) and 16-month-old (solid column) mice were cultured in the presence of anti-CD3 antibody for 3 days and pulsed with ^{3}H-TdR (left panel). CD4$^+$ T cells from 16-month-old mice were separated into CD44^{low} (light grey column), PD-1$^-$ CD44^{high} (dark gray column) and PD-1$^+$ CD44^{high} (solid column) populations and cultured similarly (middle panel). *, $P < 0.05$; **, $P < 0.01$. The latter two populations were analyzed for the expression TCRβ and CD3 (right panel). (C) Sorted PD-1$^-$ and PD-1$^+$ CD44^{high} CD4$^+$ T cells from aged mice were transferred into Rag2$^{-/-}$ (upper left) or γ-ray-irradiated CD45.1 B6 (lower left) mice intravenously, and 6 days later the donor T cells (boxed) in the pooled lymphoid tissues were assessed. Percent donor cells of total and CD4$^+$ T cells in Rag2-/- and irradiated B6 recipients are indicated, respectively. Similar results were obtained in 3 recipients. T cells from normal aged B6 mice were three-color analyzed with the indicated antibodies (right). (D) The proportions of total CD44^{high} (dotted lines), PD-1$^-$ CD44^{high} (open circles) and PD-1$^+$ CD44^{high} (closed circles) T cells in CD4$^+$ T cell population at various ages are plotted.

Fig. 2. PD-1$^+$ MP CD4$^+$ T cells show a distinctive genetic signature. (A) CD4$^+$ T cells from 2-month-old B6 mice as well as sorted CD44^{low} (I), PD-1$^-$ CD44^{high} (II) and PD-1$^+$ CD44^{high} (III) CD4$^+$ T cell populations from 18-month-old B6 mice were stained for

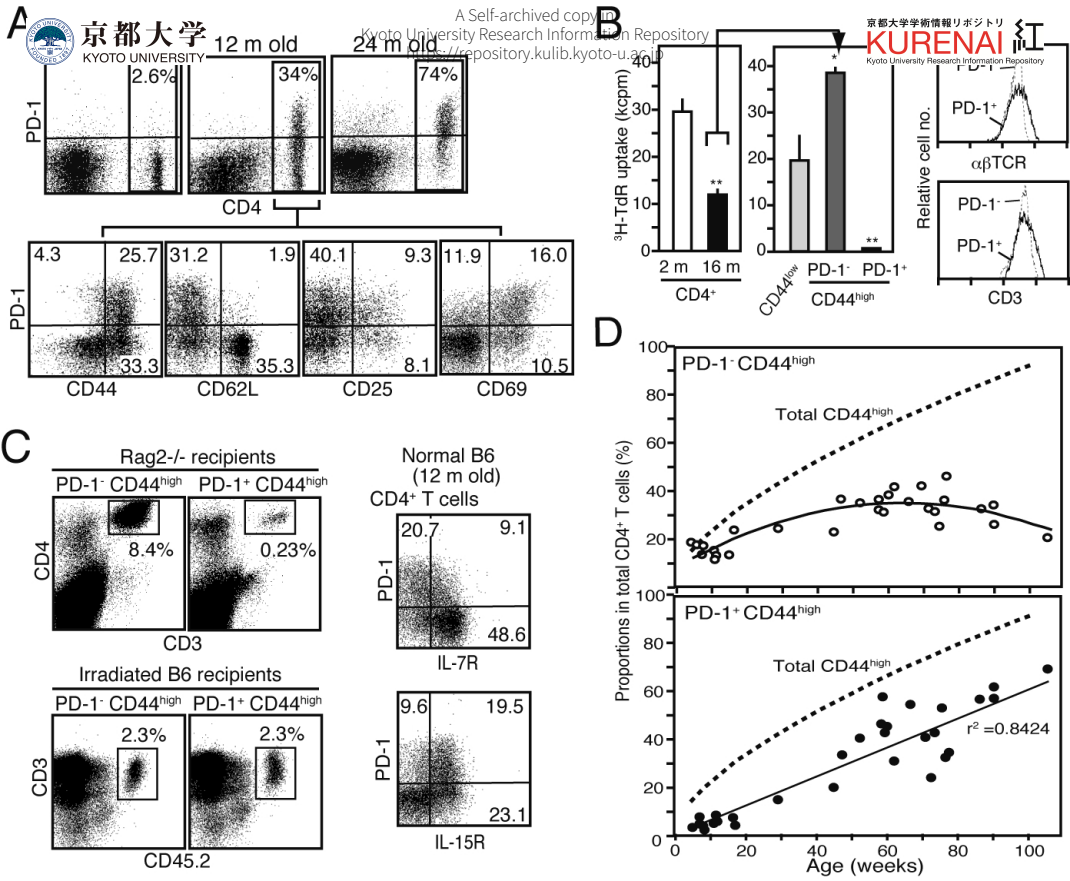
SA-β-Gal. The arbitrary signal units of about 100 cells in each group are plotted. (B) Sorted CD44^{low} (I), PD-1^{-} CD44^{high} (II) and PD-1^{+} CD44^{high} (III) CD4^{+} T cell populations from aged mice were analyzed for the expression of 43 genes with qRT-PCR. Relative transcripts in the three populations are indicated. (C) PD-1^{-} and PD-1^{+} CD44^{high} CD4^{+} T cells sorted from 2- and 12-month-old mice were analyzed for the transcripts of 16 genes with qRT-PCR. The ratios of transcripts (PD-1^{+}/PD-1^{-} CD44^{high} CD4^{+} T cells) for each gene in the 2 groups are plotted against each other. (D) Sorted PD-1^{-} (open columns) and PD-1^{+} (solid columns) CD44^{high} CD4^{+} T cells from aged mice were cultured with or without anti-CD3 plus anti-CD28 antibodies. OPN in the culture supernatants was assessed on days 1 and 3 with ELISA (upper). Aliquots of the cells were harvested on day 1, and the transcripts of the indicated genes were assessed with qRT-PCR (lower). N.D.; not detectable.

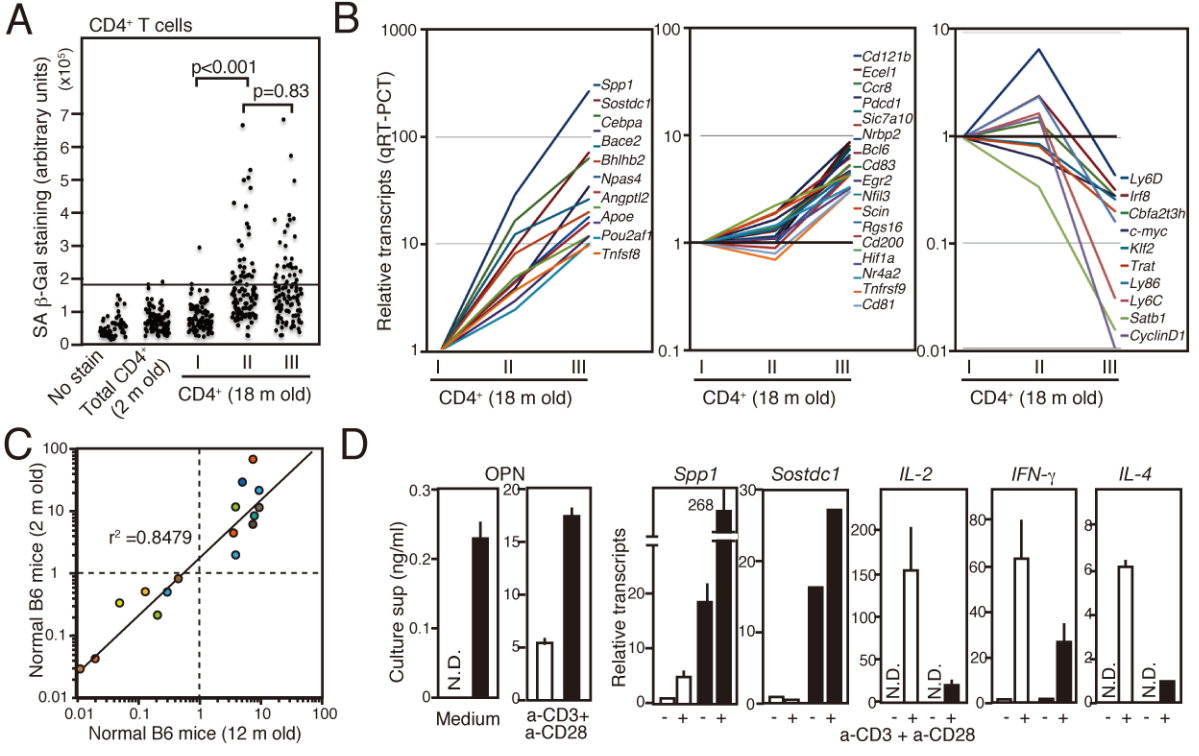
Fig. 3. Expression of *Cebpa* in regular CD4^{+} T cells recapitulates the functional features of PD-1^{+} MP CD4^{+} T cells. (A) Sorted PD-1^{-} and PD-1^{+} CD44^{high} CD4^{+} T cells from aged mice were immunoblotted with the indicated antibodies. (B) Sorted CD4^{+} T cells from young mice were cultured with anti-CD3 plus anti-CD28 antibodies and infected with an empty (open columns) or *Cebpa*-containing (solid columns) retrovirus (MIG) on day 1. GFP^{+} T cells were sorted on day 3 and assessed for transcripts of the indicated genes with qRT-PCR (upper). The cells were cultured in the presence of IL-2 for 2 more days and pulsed with ^{3}H-TdR (lower). **, $P < 0.01$; *, $P < 0.05$.

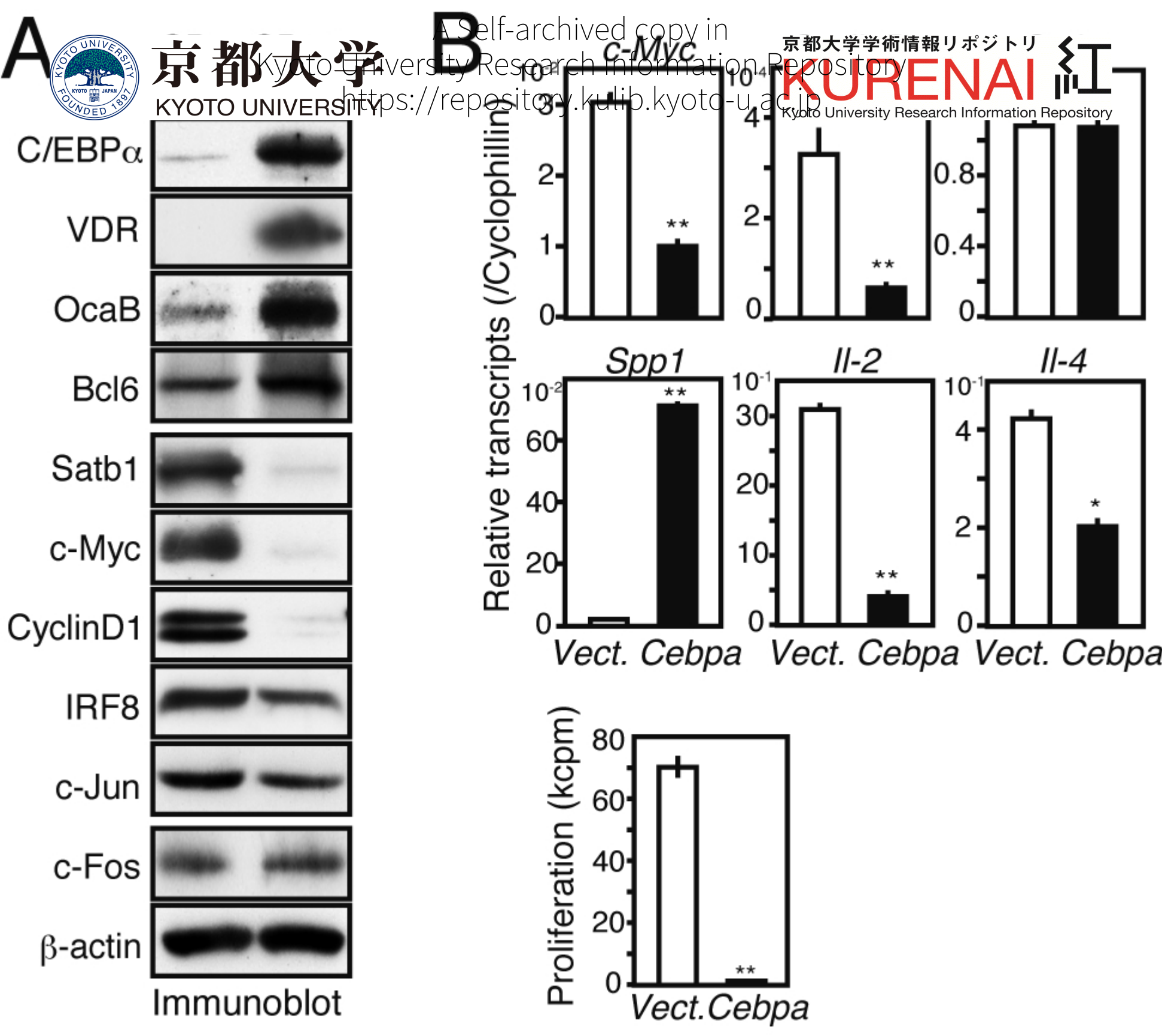
Fig. 4. Senescence-related PD-1$^+$ MP CD4$^+$ T cells are generated robustly during leukemia. (A) Young B6 mice were inoculated intravenously with BA-1 leukemia cells, and the cells of BM, spleen and blood from 2-week posttransplant mice were three-color analyzed with the indicated antibodies. Circles indicate the leukemia cells. (B) CD4$^+$ T cells from pre- (light gray column) and post-transplant (dark gray column) mice were cultured with anti-CD3 antibody for 3 days to assess DNA synthesis (upper left). The CD4$^+$ T cells from leukemia-bearing mice were separated into PD-1$^-$ (open column) and PD-1$^+$ (solid columns) CD44^{high} populations and cultured similarly (upper middle). The indicated cytokines in the culture supernatants were assessed with ELISA (upper right and lower). *, $P < 0.01$, *, $P < 0.05$. (C) DNA microarray data of PD-1$^-$ and PD-1$^+$ CD44^{high} CD4$^+$ T cells from normal aged and BA-1-bearing young mice were subjected to the cluster analysis with the combinations of PD-1$^+$ [aged]/PD-1$^-$ [aged] (I), PD-1$^+$ [leukemic]/PD-1$^-$ [aged] (II) and PD-1$^+$ [leukemic]/PD-1$^-$ [leukemic] (III) (upper panel). The ratios of transcripts (PD-1$^+$/PD-1$^-$ populations) of 16 genes in the two groups are plotted against each other (lower). (D) The proportions of leukemia cells in the blood (solid circles), PD-1$^+$ CD44^{high} cells in the splenic CD4$^+$ T cells (open squares) as well as the TCR-mediated proliferation of CD4$^+$ T cells (open circles) were assessed at various days after the inoculation with BA-1 cells. The means of 3 mice are shown.

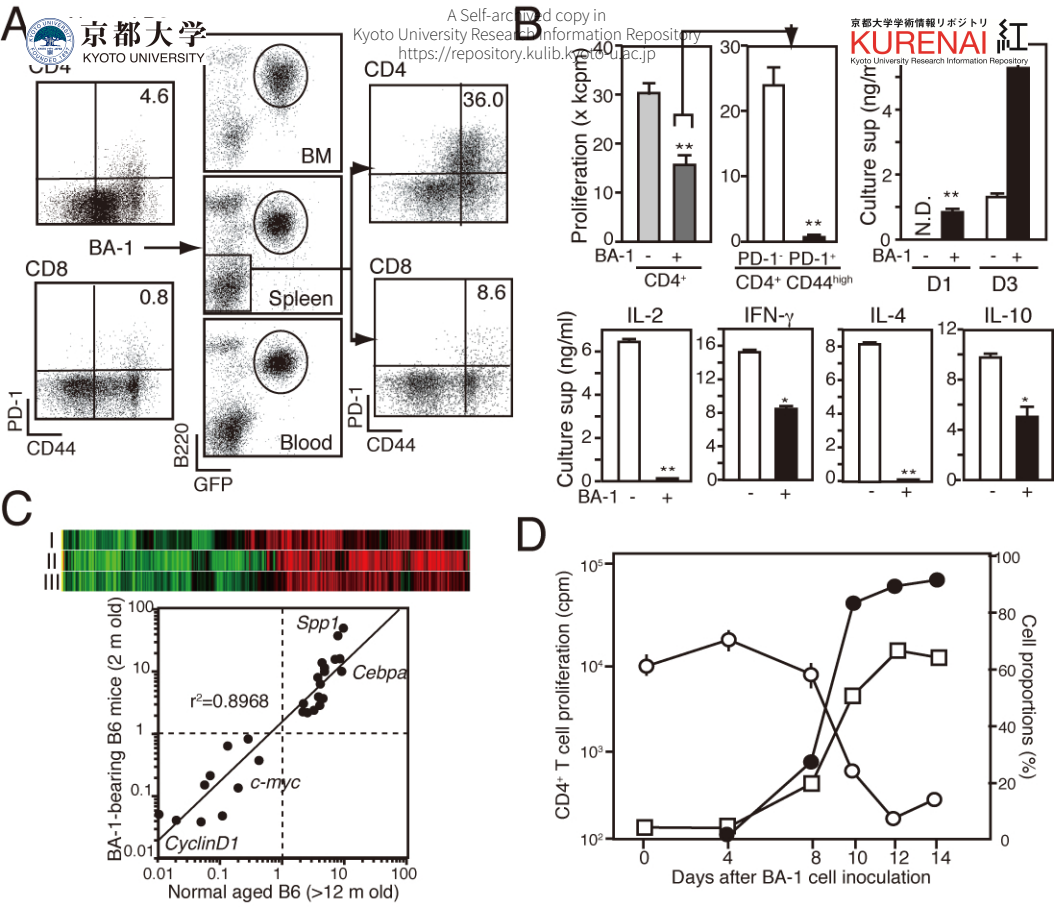
Fig. 5. Emergence of PD-1$^+$ CD4$^+$ T MP cells is correlated with the clonal T-cell immunodepression in senescence and during leukemia. (A) Spleen cells from normal young (open columns), aged (grey columns) and leukemia-bearing young (solid

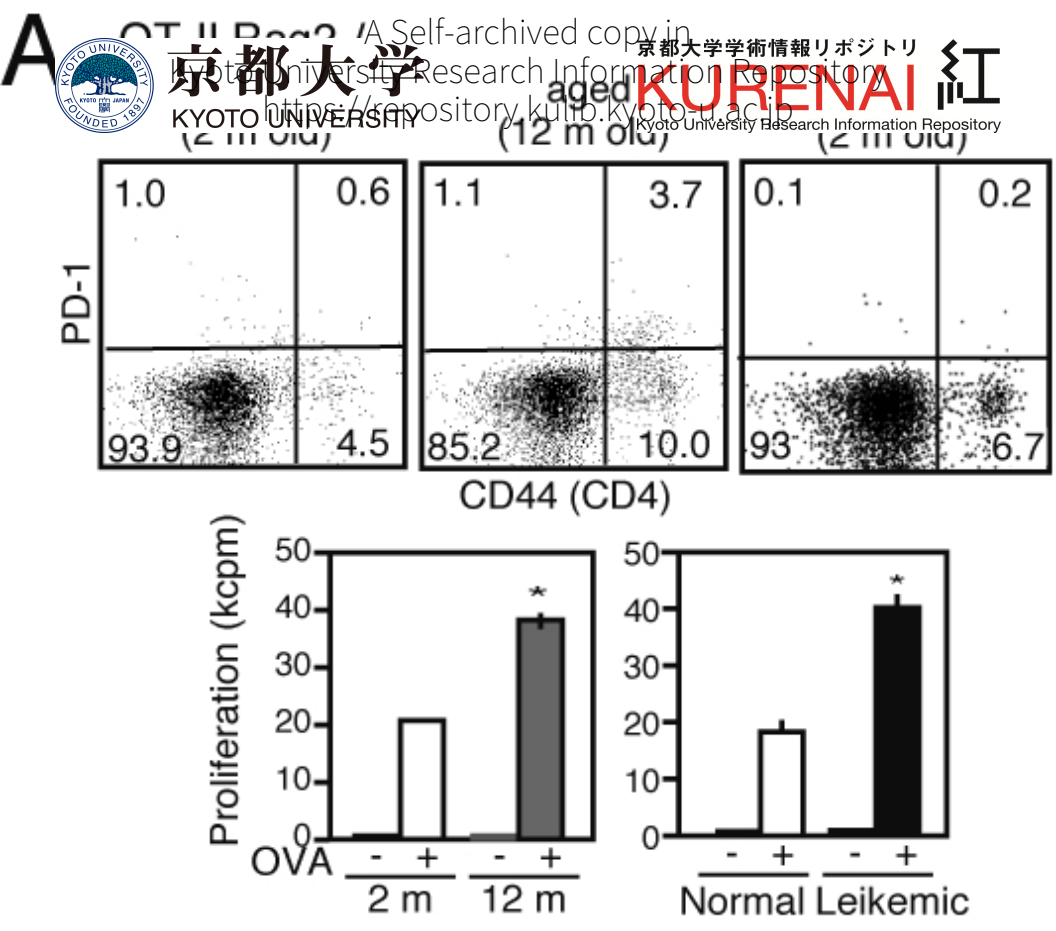
columns) OT-II/Rag2$^{-/-}$mice were three-color analyzed with the indicated antibodies. The CD4^{+} cells were sorted from each group and cultured in the presence of irradiated B6 spleen cells with or without OVA for 3 days. *, $P < 0.01$. (B) Identical experiments were performed in OT-II/B6 mice, except that PD-1^{-} (light gray columns) and PD-1^{+} (dark gray columns) CD44^{high} CD4^{+} T cells were examined separately in aged mice. *, $P < 0.05$; **, $P < 0.01$.

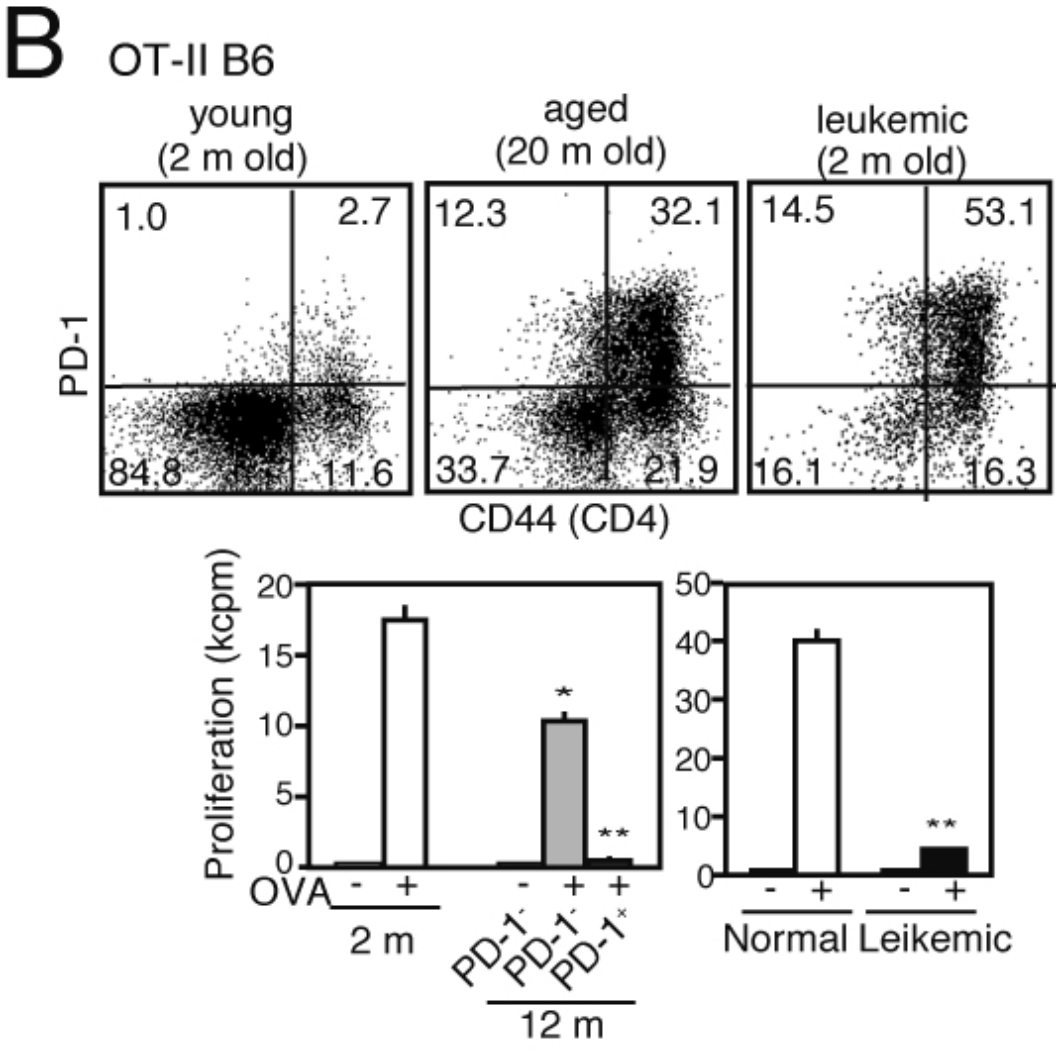












SI Materials and Methods

Cell lines. BA-1 ($B220^+$ pro-B cell leukemia) cell line was established from the bone marrow of SPA-$1^{-/-}$ mice by *bcr-abl (GFP)* transduction. Wo-1 (pro-T cell leukemia) cell line was established from the T-cell leukemia developed in the recipients of C3G-transfected Spa-1-/- bone marrow cells. These leukemia cells in blood were monitored by flow cytometry for GFP.

Flow cytometry. The following antibodies were used: antibodies to CD3ε, CD4, CD8, CD25, CD44, CD45.1, CD45.2, CD62L, CD69, B220, TCR-Vβs, Foxp3, IL-7Rα, IL-15Rβ, anexin V (eBioscience), CD121b and TCRβ (BD Pharmingen).

Quantitative RT-PCR analysis. Total RNA was extracted from the purified $CD4^+$ T cell subpopulations with a NucleoSpin RNA kit (Macherey-Nagel) followed by cDNA synthesis with SuperScript III (Invitrogen). The primer pairs are listed in Table S2. The transcripts of each gene were normalized to those of cyclophilin.

Immunoblotting. Cells were lysed with ice-cold RIPA lysis buffer containing protease inhibitors, and the extracts were subjected to immunoblotting. Antibodies included β-actin, Bcl6, C/EBPα, ERK2, pERK1/2, cFos, IRF8, OcaB, Satb1, VDR (Santa Cruz), c-Jun, c-Myc and Cyclin D1 (Cell Signaling Technology).

SA-β-Gal staining. The microscopic images of SA-β-Gal staining were stored in Tiff

files, and blue color signal per cell was quantified with the use of MetaMorph software (Molecular Devices).

DNA microarray and clustering analysis. ; PMT levels were adjusted to achieve 0.1%-0.5% pixel saturation; and each tiff image was analyzed with GenePix Pro 6.0 (Molecular Devices). Data were filtered to remove low-confidence measurements and globally normalized per array such that the median log2 (Cy3/Cy5 fluorescence ratio) was zero after normalization.

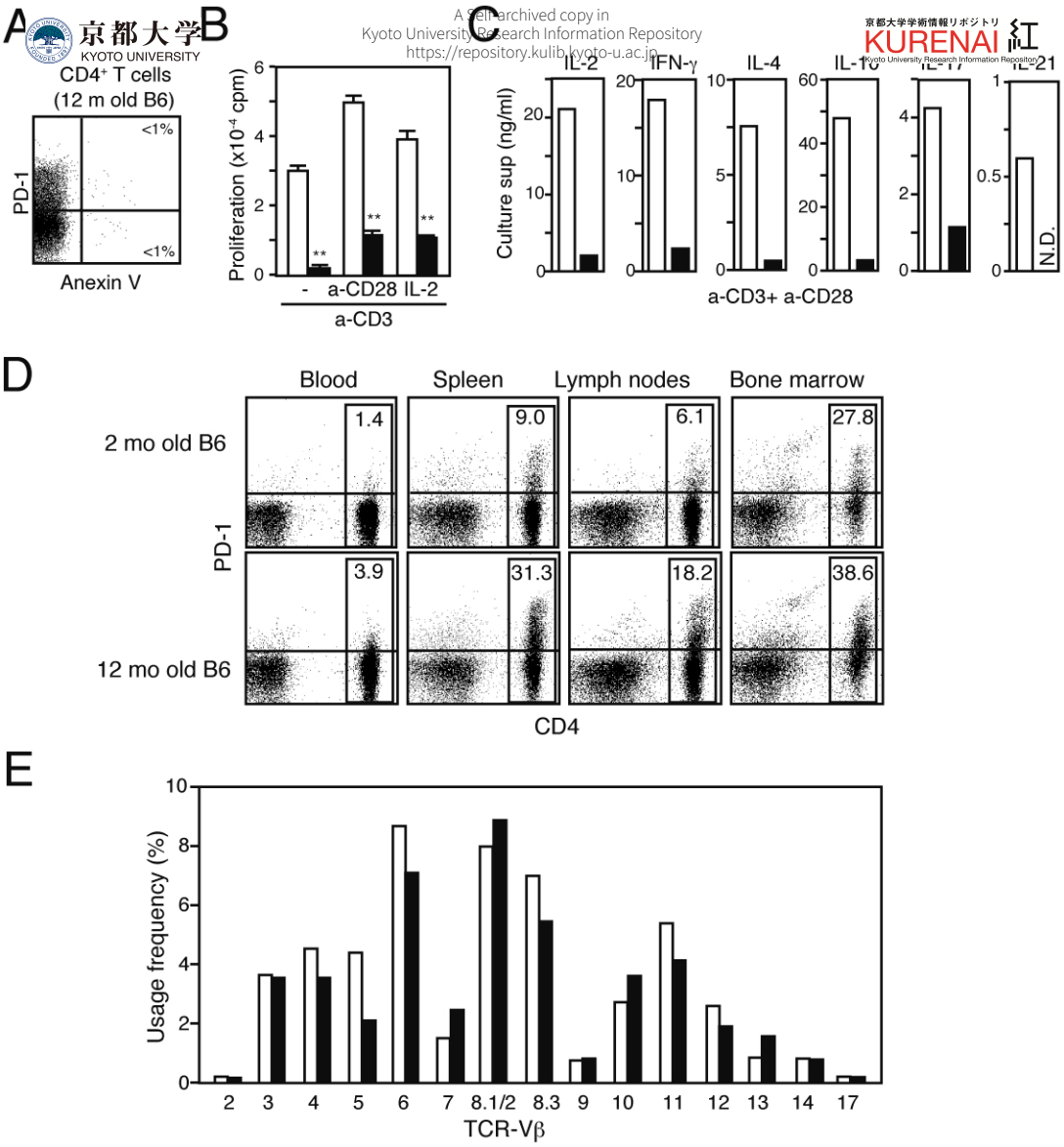
Legends for Supporting Figures

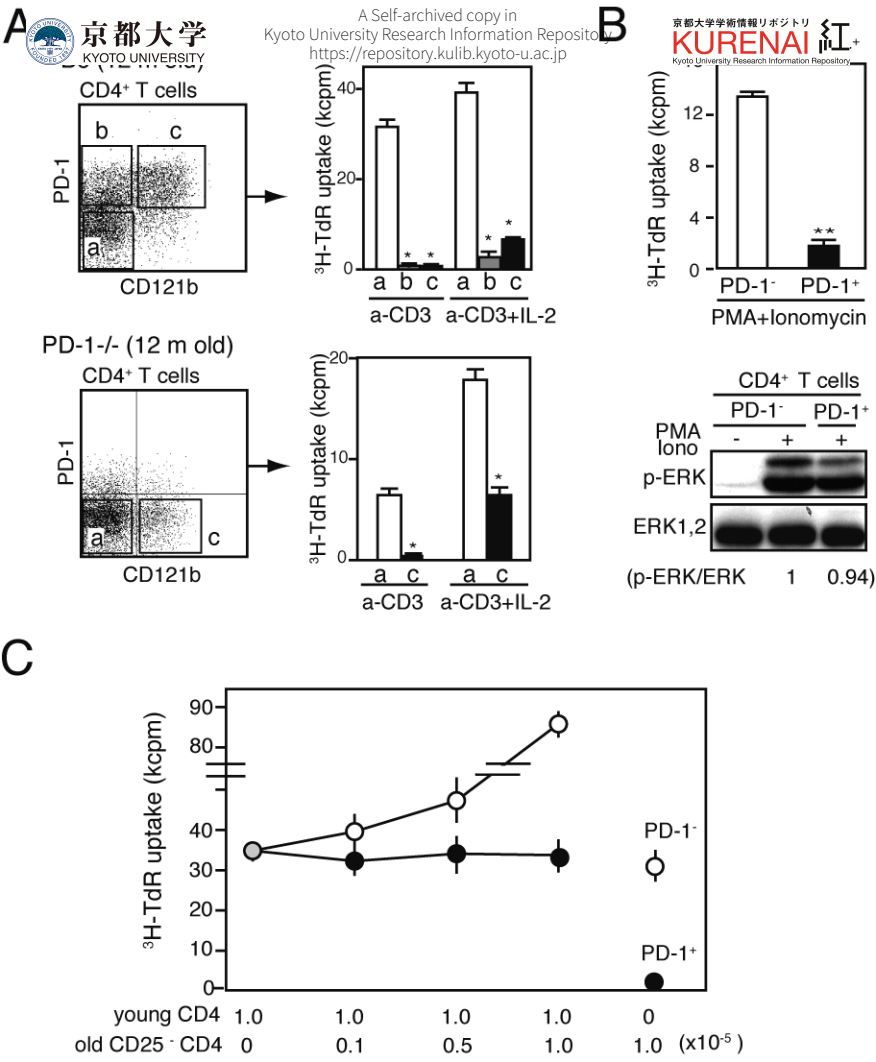
Fig. S1. Proliferation capacity, organ distribution and TCRβ repertoire of PD-1$^+$ MP CD4$^+$ T cells. (*A*) Spleen cells from 12 month old B6 mice were 3-color analyzed with the indicated antibodies. (B) PD-1$^-$ (open columns) and PD-1$^+$ (closed columns) CD44^{high} CD4$^+$ T cells sorted from 12-month old B6 mice were cultured in the presence of anti-CD3 antibody (5 μg/ml) with or without anti-CD28 antibody (2.5 μg/ml) or IL-2 (100 U/ml) for 3 days and pulsed with ^{3}H-TdR. **; *P*<0.01. (C) PD-1$^-$ (open columns) and PD-1$^+$ (solid columns) CD44^{high} CD4$^+$ T cells sorted from aged mice were cultured in the presence of anti-CD3 plus anti-CD28 antibodies for 3 days, and the indicated lymphokines were assessed with ELISA. N.D.; not detectable. (D) Cells from the blood and indicated lymphoid tissues of 2- and 12-month old B6 mice were three-color analyzed with anti-CD3, anti-PD-1, and anti-CD4 antibodies. The profiles in a CD3$^+$ gate are indicated. (E) PD-1$^-$ (open columns) and PD-1$^+$ (closed columns) CD44^{high} CD4$^+$ T cells sorted from 12-month old B6 mice were analyzed with a set of anti-TCRβ chain antibodies with FACSCaliber.

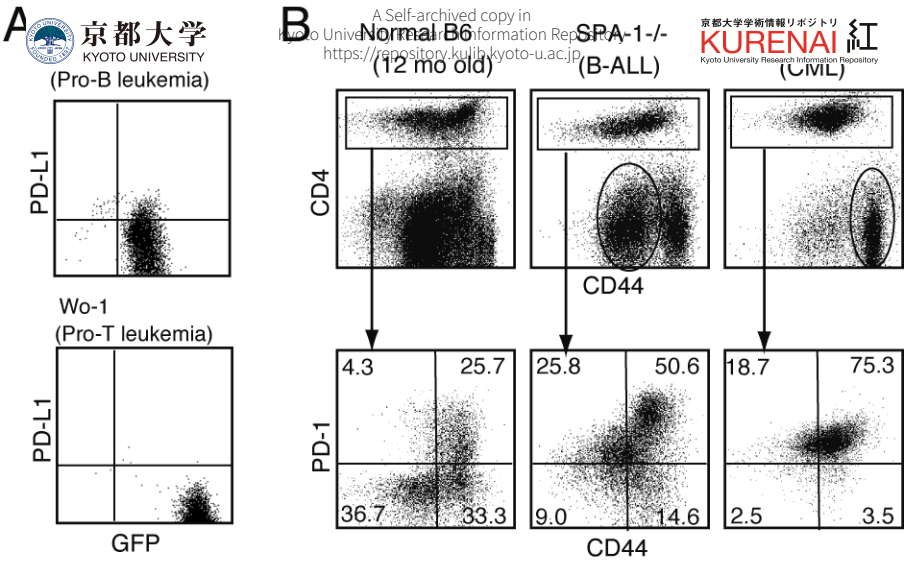
Fig. S2. PD-1$^+$ MP CD4$^+$ T cells are intrinsically defective for TCR-mediated proliferation. (A) Spleen cells from B6 and PD-1$^{-/-}$ mice at the age of 12 months were three-color analyzed with the indicated antibodies (left). PD-1$^-$ CD121b$^-$ (a, open column), PD-1$^+$ CD121b$^-$ (b, gray column) and CD121b$^+$ (c, solid column) CD4$^+$ T cells were sorted and cultured in the presence of anti-CD3 antibody with or without IL-2 for 3 days (right). *, P < 0.01. (B) PD-1$^-$ (open column) and PD-1$^+$ (closed column) CD44$^+$

CD4$^+$ T cells from aged B6 mice were cultured in the presence of PMA plus ionomycin for 10 min to detect ERK activation and for 3 days to assess the proliferation. Relative p-ERK/ERK ratio was assessed by a densitometry. **, $P < 0.01$. (C) PD-1$^-$ (open circles) and PD-1$^+$ (closed circles) CD25$^-$ CD4$^+$ T cells sorted from aged mice were cocultured with CD4$^+$ T cells from young mice at various ratios in the presence of anti-CD3 plus and-CD28 antibodies for 3 days.

Fig. S3. PD-1$^+$ MP CD4$^+$ T cells are increased as Spa-1$^{-/-}$ mice spontaneously develop frank leukemia. (A) Expression of PD-L1 was analyzed in the leukemia cell lines (BA-1 pro-B cell and Wo-1 pro-T cell leukemia), both of which are capable of inducing the rapid generation of PD-1$^+$ MP CD4$^+$ T cells in vivo. (B) Spleen cells from Spa-1$^{-/-}$ mice with frank leukemia (B-ALL and CML) and from age-matched (12 month-old) control B6 mice were 3-color analyzed with the indicated antibodies. Circles indicate the leukemia cells. (C) The proportions of PD-1$^+$ MP CD4$^+$ T cells in the spleen cells from Spa-1$^{-/-}$ mice at various ages are plotted. Open circles indicate the mice with no evidence of leukemia and closed circles the mice with frank leukemia.







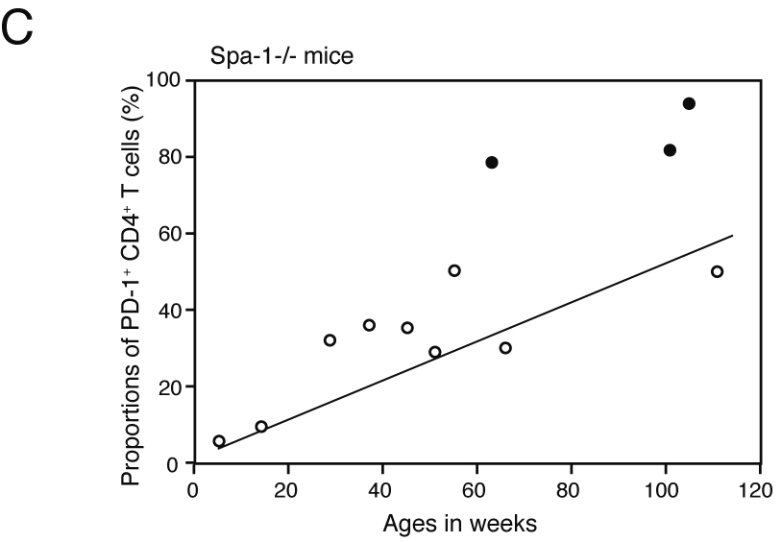


Table S1. DNA microarray analysis

[Overexpressed genes]		Normal aged B6 MP CD4+ T cells		
		PD-1(-)	PD-1 (+)	Ratio
Transcription factors				
Batf	basic leucine zipper transcription factor, ATF-like	883	2824	3.2
Nfil3	nuclear factor, interleukin 3, regulated	237	740	3.1
Zdhhc2	zinc finger, DHHC domain containing 2	145	388	2.7
Hif1a	hypoxia inducible factor 1, alpha subunit	292	1003	3.4
Plagl1	pleiomorphic adenoma gene-like 1	143	826	5.8
Tox	thymocyte selection-associated HMG box gene	684	2017	3.0
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha (Cebpa)	183.4	970.5	4.8
Pou2af1	POU domain, class 2, associating factor 1 (Pou2af1)	265.8	948.4	3.2
Bcl6	B-cell leukemia/lymphoma 6 (Bcl6)	1147.3	3914.7	3.1
Egr2	early growth response 2 (Egr2)	765.1	1622.8	1.9
Vdr	vitamin D receptor (Vdr), mRNA	183.8	893.1	4.4
Membrane proteins				
Pdcd1	programmed cell death 1	107	646	6.0
Cacna1d	calcium channel, voltage-dependent, L type, alpha 1D	40	161	4.0
Cd121b	interleukin 1 receptor, type II	79	584	7.4
Ptger2	prostaglandin E receptor 2 (subtype EP2)	63	241	3.8
Tnfsf8	tumor necrosis factor (ligand) superfamily, member 8	333	959	2.9
Cxcr4	chemokine (C-X-C motif) receptor 4	168	874	5.2
Cd200	Cd200 antigen	204	678	3.3
Cd83	CD83 antigen	310	1491	4.8
Blr1	Burkitt lymphoma receptor 1	529	1757	3.3
Cd81	CD 81 antigen (Cd81)	3426.2	11450.9	3.0
Cytokines/secreted proteins				
Sostdc1	sclerostin domain containing 1	397	5375	13.5
Ccl3	chemokine (C-C motif) ligand 3 [Source:MarkerSymbol;Acc:MGI:98260]	107	646	6.0
Ccl4	chemokine (C-C motif) ligand 4	203	926	4.6
Il4	interleukin 4	176	421	2.4
Il21	interleukin 21	173	1017	5.9
Stfa1	stefin A3	19	229	12.3
Spp1	pogo transposable element with ZNF domain	96	1418	14.7
Esm1	endothelial cell-specific molecule 1	179	530	3.0
Cytoplasmic proteins				
Cdkn2b	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	75	294	3.9
Sccpdh	saccharopine dehydrogenase (putative)	92	461	5.0

Rgs16	regulator of G-protein signaling 16	97	492	5.1
Srxn1	sulfiredoxin 1 homolog (S. cerevisiae)	135	361	2.7
Pfn2	profilin 2	66	241	3.7
Stk39	serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	502	1154	2.3
Cxxc5	ring finger protein 128	103	266	2.6
Stx11	syntaxin 11	325	1241	3.8
Tbc1d4	TBC1 domain family, member 4	551	1696	3.1

[Underexpresssed genes]

Transcription factors

Cbfa2t3h	core-binding factor, runt domain, alpha subunit 2	385	54	0.1
Jun	Jun oncogene	466	114	0.2
Irf8	interferon regulatory factor 8	607	73	0.1
Satb1	special AT-rich sequence binding protein 1	912	83	0.1
Cd74	Mus musculus CD74 antigen	8101.8	2714.8	0.3

Membrane proteins

Cd226	CD226 antigen (Cd226)	309.4	18.5	0.1
Cd74	Mus musculus CD74 antigen	8101.8	2714.8	0.3
Cd8b1	CD8 antigen, beta chain 1	451	164	0.4
H2-Ob	histocompatibility 2, O region beta locus	686	332	0.5
Sell	selectin, lymphocyte	695	163	0.2
Il7r	interleukin 7 receptor	1039	470	0.5
Ccr7	chemokine (C-C motif) receptor 7	238	95	0.4
Trat1	T cell receptor associated transmembrane adaptor 1	1144	309	0.3
Cd72	CD72 antigen	477	130	0.3
Ccr2	chemokine (C-C motif) receptor 2	660	188	0.3
Klrd1	killer cell lectin-like receptor, subfamily D, member 1	235	43	0.2
H2-DMb2	histocompatibility 2, class II, locus Mb1	560	90	0.2
Cd7	CD7 antigen	334	133	0.4
Ifitm1	interferon induced transmembrane protein 1	303	44	0.1
Cd8a	CD8 antigen, alpha chain	167	52	0.3
Klrc1	killer cell lectin-like receptor subfamily C, member 1	281	33	0.1
Lair1	leukocyte-associated Ig-like receptor 1	142		
Cd74	CD74 antigen	13810	2083	0.2
Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide	968	70	0.1
Amica1	adhesion molecule, interacts with CXADR antigen 1	474	36	0.1
Ly6d	lymphocyte antigen 6 complex, locus D	412	54	0.1
Ly6c1	lymphocyte antigen 6 complex, locus C1	1105	24	0.0
H2-Eb1	histocompatibility 2, class II antigen E beta [Source:MarkerSymbol;Acc:MGI:95901]	8877	453	0.1
Siglech	sialic acid binding Ig-like lectin H	633	17	0.0

	[Source:MarkerSymbol;Acc:MGI:2443256]			
Mgl1	macrophage galactose N-acetyl-galactosamine specific	154		
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	6391	268	0.0
Igl-V1	immunoglobulin lambda chain, variable 1	258		
Ly86	lymphocyte antigen 86	495	16	0.0
H2-Aa	histocompatibility 2, class II antigen E alpha	14275	391	0.0
Cytokines/secreted proteins				
Cfp	complement factor properdin	428	107	0.2
Il1b	interleukin 1 beta	467	27	0.1
Cytoplasmic proteins				
Hck	hemopoietic cell kinase	742	174	0.2
Txk	TXK tyrosine kinase	1112	226	0.2
Prkcn	protein kinase C, nu	169	17	0.1
Ncf1	neutrophil cytosolic factor 1	366	38	0.1
Ctsh	cathepsin H	583	35	0.1
Pld4	phospholipase D family, member 4	667		
Unc93b1	unc-93 homolog B1 (C. elegans)	425	163	0.4
Nedd4	neural precursor cell expressed,	395	111	0.3
Rnf130	ring finger protein 130	316	69	0.2
Plac8	placenta-specific 8	1943	58	0.0
Tyrobp	TYRO protein tyrosine kinase binding protein	2125	65	0.0
Pld4	phospholipase D family, member 4	667		
Cell cycle-related proteins				
Ccnd1	cyclin D1 (Ccnd1)	1818.8	99.0	0.1

Table S2. Primer sequences for quantitative PCR

genes	sense	antisense
Angptl2	CTGGACAGGGACCATGATGT	GGAGTGAGCACAGGCGTTAT
Cbfa2t3h	CTGACTGTCATCAACCAGCAA	TTACAGCCACTGCACGTCTC
Ccnd1	GAACAAGCTCAAGTGGAACC	CTTCAATCTGTTCCTGGCAG
Ccr8	AGAAGAAAGGCTCGCTCAGA	GGCTCCATCGTGTAATCCAT
Cebpa	TGAGAAAAATGAAGGGTGCAG	CGGGATCTCAGCTTCCTGT
c-myc	CGAAACTCTGGTGCATAAACTG	GAACCGTTCTCCTTAGCTCTCA
Cxcr6	AAGCTACTGGGCTTCTCTTCTG	CCCATCGTACAGAGCTGACTC
Cyclophilin	GACGAAGGTAGCCAGTCACAAG	AATCAGGCCTGTGGAATGTGAG
Ifng	ATCTGGAGGAACTGGCAAAA	TTCAAGACTTCAAAGAGTCTGAGG
Il1r2	CCCATCCCTGTGATCATTTC	GCACGGGACTATCAGTCTTGA
Il2	GCTGTTGATGGACCTACAGGA	TTCAATTCTGTGGCCTGCTT
Il21	TCATCATTGACCTCGTGGCCC	ATCGTACTTCTCCACTTGCAATCCC
Il4	GAGAGATCATCGGCATTTTGA	TCTGTGGTGTTCTTCGTTGC
Irf8	CCAACCAGTTCATCCGAGA	GAATGAGTTTGGAGCGCAAG
Klf2	CTAAAGGCGCATCTGCGTA	TAGTGGCGGGTAAGCTCGT
Ly6c	TCTTGTGGCCCTACTGTGTG	GCAATGCAGAATCCATCAGA
Pdcd1	CTACCTCTGTGGGGCCATC	GAGGTCTCCAGGATTCTCTCTGT
Pou2af1	CCTCCTCGGTGTTGACCTAT	CGGGTGTAGCAGTGCTTCTT
Satb1	ACTGAAACGAGCCGGAATC	CGGAGGATTTCAGAAAGCAA
Slc24a3	GCCTCATTGTAGCCAGACAAG	ACGTTGCTCCCAATGGAAT
Soscdc1	AACAGCACCCTGAATCAAGC	CAGCCCACTTGAACTCGAC
Spp1	CCCGGTGAAAGTGACTGATT	TTCTTCAGAGGACACAGCATTC
Vdr	CACCTGGCTGATCTTGTCAGT	CTGGTCATCAGAGGTGAGGTC
Bace2	CCTGAGAGATGAGAATGCCAGT	ATCATGGGCTGAATGTAGAGC
Bhlhb2	TGAAGCACGTGAAAGCATTG	TTTCTTCCCGACAAATCACC
Npas4	AGGGTTTGCTGATGAGTTGC	CCCCTCCACTTCCATCTTC
Grail	GTAACCCGCACACCAATTTC	GTGAGACATGGGGATGACCT
Apoe	GACCCTGGAGGCTAAGGACT	AGAGCCTTCATCTTCGCAAT
Tnfsf8	GAGGATCTCTTCTGTACCCTGAAA	TTGGTATTGTTGAGATGCTTTGA
Cd121b	CCCATCCCTGTGATCATTTC	GCACGGGACTATCAGTCTTGA
Ecel1	GCCCAACAAGAATCAAATGG	CCCCCGTAGTTCAGAGACTG
Bir1	GGAGGGTACCACTCACATGG	TTGCCTGCTAACTTCCCCTA
Nrbp2	CTGAGTGACCCCAACATGC	CGGTGGAAGAGGAGGTTGT
Bcl6	TTCCGCTACAAGGGCAAC	CAGCGATAGGGTTTCTCACC
Cd83	TGGTTCTGAAGGTGACAGGA	CAACCAGAGAGAAGAGCAACAC
Egr2	GTGCCAGCTGCTATCCAGAAG	GGCTGTGGTTGAAGCTGGAG
Nfil3	AGGCCGATGAGGGTGTAGT	GCCCTTAGGGACCTGTTGTT
Batf	AGCTTCAGCCGCTCTCCT	GCAGCGATGCGATTCTTC
Scin	CACAGTTCTCCGCAGATGG	CACTGTTCTCTACACGCCAGA
Rgs16	GGCCAGTAAGCATAACAAAGAGA	TCAGCAGCAAATCGAAAGAC
Cd200	CTCCACCTACAGCCTGATTTG	CCTGGGTCACCACTTCCA

Hif1a	GCACTAGACAAAGTTCACCTGAGA	CGCTATCCACATCAAAGCAA
Nr4a2	TCAGAGCCCACGTCGATT	TAGTCAGGGTTTGCCTGGAA
Tnfrsf9	TGTGACTCCAGAGGGAGGAC	AGCAGCAAAGCCGATGTC
Cd81	CCTGGAACTGGGAAACAAAC	GCTCCCACAGCAATGAGAAT
Ly6d	TCTGCTCGTCCTCCTTGTCT	GTGCACACGTGACATCGAA
Trat	AGGAAGTGGCTGCACCTG	CCAGTGAGGCATAGCACATC
Ly86	ATTCTGAACTACTCCTATCCCCTTT	GGCCGGCATAGTATATCTGTTCT